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Research on the Mechanism of Cuproptosis-related Gene LIAS in Regulating Nasopharyngeal Carcinoma

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Abstract

Background

Nasopharyngeal carcinoma (NPC) is a malignant tumor with a high incidence in endemic regions, and the identification of novel therapeutic targets remains essential. Lipoic acid synthetase (LIAS), a key regulator of mitochondrial metabolism and a cuproptosis-related gene, has been implicated in tumor progression; however, its role in NPC remains unclear.

Results

LIAS expression was evaluated in NPC tissues and cell lines, and its effects on proliferation, migration, invasion, energy metabolism, and Elesclomol-$CuCl_2$ (ES-Cu)-induced cuproptosis were assessed *in vitro*. Transcriptomic sequencing, co-immunoprecipitation (Co-IP), and molecular simulations were used to explore the mechanism, with *in vivo* validation in a xenograft model. LIAS was downregulated in NPC tissues and cell lines. LIAS overexpression suppressed malignant behaviors, altered energy metabolism, and increased sensitivity to ES-Cu-induced cuproptosis. Transcriptomic analysis identified LHPP as a downstream target. LIAS positively regulated LHPP expression and interacted with LHPP. LHPP overexpression enhanced the inhibitory effects of LIAS, whereas the LHPP R157A mutation partially attenuated these effects. *In vivo*, LIAS overexpression combined with ES-Cu further inhibited tumor growth and enhanced cuproptosis-related changes.

Conclusion

LIAS suppresses NPC progression and enhances sensitivity to ES-Cu induced cuproptosis, potentially through regulation of the LIAS-LHPP axis and cellular energy metabolism. These findings provide new insights into the molecular mechanisms of NPC and the potential application of cuproptosis based therapeutic strategies.

1 Introduction

Nasopharyngeal carcinoma (NPC) is a malignant tumor originating from the epithelial cells of the nasopharyngeal mucosa. Genetic factors, Epstein-Barr virus (EBV) infection, and environmental risk factors are the primary causes underlying the occurrence and progression of NPC [1, 2]. As of 2022, NPC affected approximately 120,000 individuals globally and caused about 70,000 deaths [3]. Owing to the occult nature of the disease, over 75% of patients are diagnosed at an advanced stage at their first presentation [4]. Therefore, identifying potential therapeutic targets for NPC is of great research significance.

The balance between cell death and proliferation is crucial in mammalian somatic cells [5]. In NPC, this balance is often disrupted [6]. With advances in research, cuproptosis has attracted increasing attention as a new way of regulated cell death. Its mechanism is different from other known cell death pathways [7]. Cuproptosis is a copper-dependent, mitochondrial lipoylation-driven form of regulated cell death, which has been identified as an important regulator in cancer initiation and progression [8]. Lipoic acid synthetase (LIAS) is a key regulatory gene in the cuproptosis pathway and plays an essential role in regulating mitochondrial energy metabolism and maintaining cellular antioxidant defense systems [9]. It has been reported that LIAS can suppress the viability and proliferation of prostate cancer cells by enhancing cuproptosis [10]. However, the expression pattern and regulatory mechanisms of LIAS in NPC remain largely unclear.

Therefore, this study integrated bioinformatics analysis with *in vitro* and *in vivo* experimental validation to investigate the role and underlying mechanisms of LIAS in NPC. LIAS expression was first evaluated in clinical tissue samples and NPC cell lines, and its association with tumor stage and cuproptosis related molecular features was subsequently assessed. Functional experiments were then performed to determine the effects of LIAS on NPC malignant behaviors and sensitivity to the cuproptosis inducer ES-Cu. Transcriptomic analysis and subsequent molecular and functional validation further identified LHPP as a downstream mediator of LIAS and investigated its involvement in cellular energy metabolism and cuproptosis. Collectively, this study provides new insights into the role of the LIAS-LHPP axis in NPC progression and ES-Cu sensitivity and provides a potential molecular basis for developing cuproptosis-based therapeutic strategies for NPC.

2 Materials and methods

2.1 Ethics approval and clinical sample collection

This study was conducted in accordance with the principles of the Declaration of Helsinki and was approved by the Ethics Committee of Chongqing General Hospital (Approval No. IIT S2025-044-01). The use of these pre-existing, de-identified materials was reviewed and approved by the ethics committee, which granted a waiver of individual written informed consent for the retrospective analysis. For any prospectively collected samples or data, informed consent was obtained from all participants.

Archived and fully anonymized NPC tissue specimens and matched clinical data were collected from 30 patients diagnosed at the Department of Otorhinolaryngology-Head and Neck Surgery of Chongqing General Hospital within the past five years. The control group comprised tissue samples from 34 patients with benign nasopharyngeal lesions (BNPL), pathologically confirmed as chronic nasopharyngeal mucosal inflammation, who were randomly selected from individuals presenting during the same period. Disease staging was performed in accordance with the Union for International Cancer Control (UICC)/American Joint Committee on Cancer (AJCC) TNM 8th edition. All patients received standard chemoradiotherapy following the Chinese Society of Clinical Oncology (CSCO) guidelines for

nasopharyngeal carcinoma. All samples were processed under strict anonymization protocols to safeguard patient privacy.

2.2 Cell

Human immortalized nasopharyngeal epithelial cell NP69 (product number: iCell-h431), human nasopharyngeal carcinoma cell C666-1 (product number: iCell-h378), HNE1 (product number: iCell-h459), and HK-1 (product number: iCell-h367) were all purchased from iCell Bioscience Inc, Shanghai.

2.3 Animals

Forty 4-week-old male specific pathogen-free (SPF) BALB/c-nu nude mice were purchased from Chengdu Yaokang Biotechnology Co., Ltd. (production license No. SCXK (Chuan) 2025-0034). After 7 days of acclimatization, the mice were randomly assigned to the indicated experimental groups and housed in a barrier facility at a density of five mice per cage. The animal room was maintained under stable temperature and relative humidity conditions with a 12-hour light/dark cycle. All animal experiments were approved by the Ethics Committee of Chongqing General Hospital (Approval No. KY S2025-019-01) and performed in accordance with the ARRIVE guidelines and relevant national guidelines for animal welfare.

2.4 Immunohistochemistry (IHC) stain

Paraffin-embedded sections were deparaffinized, rehydrated, and subjected to antigen retrieval. After blocking, sections were incubated with the corresponding primary antibodies, followed by incubation with an HRP-conjugated goat anti-rabbit secondary antibody (1:100, GB23303, Servicebio). Immunoreactivity was visualized using a DAB kit (1:20, ZL1-9018, ZSGB-BIO), and nuclei were counterstained with hematoxylin. Then dehydration and sealing were performed, and the slides were observed under a microscope. The primary antibodies used were as follows: anti-Ki-67 (1:1000, HA721115, HUABIO) and anti-LIAS (1:2000; YT8133; Immunoway).

2.5 siRNA transfection

Small interfering RNAs (siRNAs) were synthesized by GenePharma (Shanghai, China). For the siRNA transfection experiments, siRNA was mixed with transfection reagent to form transfection complexes, which were then added to antibiotic-free complete medium and applied to the cells for transfection. The siRNA sequences used in this study were as follows: si-NC: sense 5’-UUCUCCGAACGUGUCACGUTT-3’, antisense 5’-UUCUCCGAACGUGUCACGUTT-3’; si-LIAS: sense 5’-GCACCUGGGAUGAAUAUAATT-3’, antisense 5’-UUAUAUUCAUCCCAGGUGCTT-3’.

2.6 Lentiviral infection

The LIAS overexpression lentiviral were constructed by GeneChem (Shanghai, China). Cells were seeded and infected at a multiplicity of infection (MOI) of 100. After 16 h of infection, the medium was replaced with complete medium, and the cells were cultured for an additional 48 h. Stable ov-LIAS cells were

established by selection with puromycin (0.5 μg/mL, HY-K1057, MCE) for approximately 15 d, with the selection medium replaced every 2–3 d.

2.7 Plasmid transfection

The wild type LHPP overexpression plasmid (ov-LHPP^{WT}) and the mutant LHPP plasmid (ov-LHPP^{MUT}), in which arginine at position 157 was substituted with alanine (R157A), were obtained from Anzhen Biotechnology (H65465, China). Cells were seeded and transfected with the indicated plasmids using Lipofectamine 3000 (Invitrogen) according to the manufacturer's instructions. Briefly, plasmids were mixed with Lipofectamine 3000 in Opti-MEM™ I reduced-serum medium to form transfection complexes, which were then added to the cells. After 6 h, the transfection medium was replaced with complete medium, and the cells were cultured for subsequent experiments.

2.8 Cell treatment

After lentiviral transduction for 48 h, cells were treated with the indicated concentrations of ES-Cu (HY-12040, MCE) according to the experimental groups and incubated at 37℃ for 48 h. For the TTM rescue experiment, cells were pretreated with 1 μM ammonium tetrathiomolybdate (TTM; HY-W076067, MCE) for 2 h before lentiviral transduction, and an equal amount of TTM was replenished during medium replacement.

2.9 Establishment of mouse models and drug treatment

Logarithmically growing cells stably expressing the indicated vectors were collected, resuspended in sterile saline at 2×10^7 cells/mL, and 100 μL of the cell suspension was subcutaneously injected into the left anterior axillary region of each nude mouse. Seven days after inoculation, mice with successful tumor formation were randomly assigned to the indicated groups.

Es-Cu was prepared by mixing Elesclomol and $CuCl_2$ solutions and diluting with 0.9% saline. Mice in the ov-NC+Elesclomol and ov-LIAS+Elesclomol groups received intraperitoneal injections at 0.25 mL/25 g every 2 d for 3 weeks, while the ov-NC and ov-LIAS groups received an equivalent volume of 0.9% saline. Body weight and tumor volume were measured every 3 d. At the end of the experiment, mice were euthanized and tissues were collected for subsequent analyses.

2.10 CCK-8

Cells in the logarithmic growth phase were taken, washed with PBS, digested with trypsin, and collected. After centrifugation, the supernatant was removed, the cells were resuspended, and the cell suspension was inoculated into 96-well plates. The cells were maintained in a constant-temperature cell culture incubator. After drug treatment, the supernatant was discarded. The CCK-8 reagent (BS350B, Biosharp, China) was prepared as a working solution and added to each well. The absorbance was measured at 450 nm using a microplate reader.

2.11 Wound healing assay

Cells were seeded in 6-well plates and cultured until a confluent monolayer formed. Aspirated the supernatant, and scratches were created using a pipette tip. The wells were gently washed twice with PBS to remove the detached cells. After transfection, cells were incubated for 48 h. Images of the scratch area were taken at 0 h and 48 h under a microscope to assess cell migration.

2.12 Transwell assay

Cells in logarithmic growth phase were seeded into 6-well plates and cultured until adherent. After transfection according to groups for 48 h, cells were serum-starved, with three replicates per group. For the migration assay, treated cells were seeded into the upper chamber of Transwell inserts, while complete medium containing 20% FBS was added to the lower chamber. For the invasion assay, the upper chamber was pre-coated with BD Matrigel before cell seeding, and subsequent seeding steps were the same as for migration assay. After 24 h incubation, liquid in the upper chamber were removed, and the remaining cells were fixed, stained with crystal violet, and counted under a microscope.

2.13 Immunofluorescence staining

Cells were fixed with 4% paraformaldehyde for 30 min and permeabilized. Cells were then blocked with serum blocking solution and incubated with primary antibody against DLAT (1:200, 13426-1-AP, Proteintech) at 4℃ overnight. cells were incubated with FITC-conjugated goat anti-rabbit secondary antibody (1:100, GB22303, Servicebio). F-actin was stained using fluorescent phalloidin (1:200, GB27303, Servicebio), and nuclei were counterstained with DAPI. Fluorescence images were captured using a fluorescence microscope, and fluorescence intensity and quantified using ImageJ software.

2.14 Copper colorimetric assay

Cells in the logarithmic growth phase were collected, and intracellular copper ion content was measured using a copper ion assay kit (Elabscience Biotechnology, Wuhan, China) according to the manufacturer's instructions.

2.15 Bioinformatics analysis

Library preparation and sequencing were performed on an Illumina NovaSeq 6000 platform. Reads were aligned to GRCh38 using HISAT2, and expression quantified with StringTie. Differentially expressed genes (DEGs) were identified using the criteria of $P < 0.05$ and $|\log2FC| > 1$. The HALLMARK_GLYCOLYSIS gene set was downloaded from the Molecular Signatures Database (MSigDB), and overlapping genes were identified using a Venn diagram. Molecular docking was performed using the HDOCK (http://hdock.phys.hust.edu.cn/) to investigate the protein-protein interaction. PyMOL (version 2.6.0a0) was used for protein structure preprocessing, distance analysis, and visualization of the amino acid residues. Molecular dynamics (MD) simulations were subsequently conducted using Gromacs 2024.4 software to evaluate the stability and interaction characteristics.

2.16 Co-immunoprecipitation

Cell lysates were prepared and normalized to equal protein concentrations. After pre-clearing with Protein A/G magnetic beads, the lysates were incubated overnight at 4°C with the indicated antibody or control IgG. Protein A/G magnetic beads were then added and incubated at 4°C for 2 h. The beads were collected by magnetic separation, washed three times with pre-cold Co-IP wash buffer, and eluted with WB/MS elution buffer at 95°C for 5 min. The immunoprecipitated proteins were analyzed by Western blotting.

2.17 Metabolic parameter measurements

Pyruvate, ATP, glucose, and lactate levels were measured using commercial assay kits according to the manufacturer's instructions and normalized to total protein content. The pyruvate (A081-1-1), ATP (A095-1-1), glucose (A154-1-1), and lactate (A019-2-1) assay kits were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

2.18 Hematoxylin and Eosin (H&E) Staining

Collected xenograft tumor tissues were fixed, paraffin-embedded, and sectioned. H&E staining was performed according to a previously published method [11]. Histopathological changes in the tumor tissues were observed under a light microscope.

2.19 Real-time fluorescence quantitative PCR (RT-qPCR)

Total RNA was extracted using the Total RNA Extraction Kit and reverse transcribed into cDNA with the PrimeScript RT Reagent Kit. RT-qPCR was performed using cDNA as the template, TB Green Premix Ex Taq as the premix. The primers used in this study were designed and synthesized by Sangon Biotech, and the sequences were as follows: F-β-actin: 5'-ATCAAGATCATTGCTCCTCCTG-3', R-β-actin: 5'-GTCATACTCCTGCTTGCTGAT-3'; F-LIAS: 5'-ATGTAGAAACAGTCCCGGAATT-3', R-LIAS: 5'-TCTCGCCTAAACCCAACATTAT-3'.

2.20 Western blot

Cell protein extracts were prepared, subjected to SDS-PAGE separation, and then transferred to PVDF membranes (Sigma-Aldrich, Shanghai, China). After blocking, the membrane was incubated with primary antibodies overnight at 4°C, followed by a secondary antibody for 2 h. The corresponding protein antibodies were as follows: LIAS (1:2000; YT8133; Immunoway), FDX1 (1:2000, 12592-1-AP, Proteintech), DLAT (1:5000, 13426-1-AP, Proteintech), Lip-DLAT (1:5000, ab58724, ABCAM), LHPP (1:1000, SC-376648, Santa Cruz), β-actin (1:50000; AC026; ABclonal), Goat Anti-Rabbit IgG (H + L) HRP (1:8000; AS014; ABclonal), HRP-conjugated Goat anti-Mouse IgG (H + L) (1:8000, AS003, ABclonal). The bands were then visualized using Torchlight' Hypersensitive ECL Western HRP Substrate (Biosharp, Shanghai, China) and imaged using the Fluorescence Image Analysis System Software V2.0.

2.21 Statistical Analysis

Statistical analysis was performed using SPSS 25.0 software (IBM Corp., USA) and GraphPad Prism 10.1.2 (GraphPad Software, USA). Data are expressed as the mean and standard deviation (SD).

Differences between two groups were analyzed using Student's t-test, while comparisons among multiple groups were evaluated using one-way analysis of variance (ANOVA). For immune infiltration comparisons involving multiple immune cell types, *P* values were adjusted using the Bonferroni method where applicable. Statistical significance was defined as $P < 0.05$.

3 Results

3.1 LIAS expression is downregulated in NPC tissues and cell lines and is associated with tumor stage

To investigate the expression pattern of LIAS in NPC, LIAS expression was examined in NPC tissues and paired benign nasopharyngeal lymphoid proliferation (BNPL) tissues. IHC analysis and RT-qPCR results demonstrated that, compared with BNPL tissues, both LIAS protein and mRNA expression levels were significantly decreased in NPC tissues (Fig. 1A, B and E). Subsequently, patients were stratified into H-LIAS and L-LIAS groups based on LIAS IHC staining results, and the association between LIAS expression and tumor stage was analyzed (Table 1). The Fisher-Freeman-Halton exact test demonstrated a significant difference in tumor stage distribution between the two groups ($P < 0.05$). Compared with the H-LIAS group, patients in the L-LIAS group exhibited a higher proportion of advanced stage tumors (stage III-IV), whereas early stage tumors (stage I-II) were more frequently observed in the H-LIAS group (Fig. 1C).

To further evaluate LIAS expression in NPC cell models, LIAS levels were examined in NP69 and NPC cell lines (HNE-1 and C666-1). Western blot showed that LIAS protein expression was significantly reduced in both HNE-1 and C666-1 cells compared with NP69 cells (Fig. 1D and F). Consistently, RT-qPCR analysis revealed that LIAS mRNA expression was significantly decreased in C666-1 cells. Although a trend of downregulated expression was observed in HNE-1 cells, the difference did not reach statistical significance (Fig. 1G). Since C666-1 is an Epstein-Barr virus (EBV)-positive NPC cell line [12], to further investigate LIAS function in NPC cells with distinct EBV backgrounds, we selected C666-1 and the EBV-negative NPC cell line HK-1 for subsequent experiments.

Table 1
Association between LIAS expression and tumor stage in patients with NPC

Variable	H-LIAS group (n = 15), n (%)	L-LIAS group (n = 15), n (%)	*P* value
Tumor stage			**0.026**
Stage Ⅰ	2 (13.33)	0 (0.00)	
Stage Ⅱ	6 (40.00)	1 (6.67)	
Stage Ⅲ	2 (13.33)	6 (40.00)	
Stage Ⅳ	4 (26.67)	8 (53.33)	
Unclassified	1 (6.67)	0 (0.00)	

Note: The unclassified case was excluded from statistical analysis. The Fisher–Freeman–Halton exact test was used to compare tumor stage distributions between the H-LIAS and L-LIAS groups. $P < 0.05$ was considered statistically significant.

3.2 LIAS overexpressing inhibits the malignant biological behaviors of NPC cells

LIAS knockdown and overexpression cell lines were constructed (Figure S1). Cell viability assays were performed in si-LIAS and ov-LIAS cells. In both cell lines, LIAS knockdown significantly enhanced cell viability, whereas LIAS overexpression markedly suppressed cell viability (Fig. 2B and C). To further explore the effect of LIAS on cell migration and invasion, wound healing assay and Transwell assay were performed (Fig. 2A and F). The wound healing assay showed that, in both C666-1 and HK-1 cells, LIAS knockdown enhanced cell migratory ability, whereas LIAS overexpression suppressed cell migration (Fig. 2D and E). Consistently, Transwell assay results demonstrated that knockdown of LIAS significantly increased the migratory and invasive capacities of both cell lines. In contrast, overexpression of LIAS markedly reduced cell migration and invasion in both C666-1 and HK-1 cells (Fig. 2G-J). These findings suggest that LIAS suppresses the malignant behavior of NPC cells.

3.3 LIAS enhances the sensitivity of NPC cells to cuproptosis inducers

To further investigate the effect of LIAS expression on the sensitivity of NPC cells to cuproptosis inducers, the optimal concentration of ES-Cu was initially determined (Fig. 3A and B). In both C666-1 and HK-1 cells, LIAS overexpression reduced cell viability, and this inhibitory effect was further enhanced with increasing concentrations of ES-Cu. At a concentration of 40 nM ES-Cu, the viability of cells in the ov-LIAS + ES-Cu group was reduced to approximately 50% in C666-1 cells and approximately 40% in HK-1 cells, showing a significant inhibitory effect while maintaining suitable experimental conditions. Therefore, 40 nM ES-Cu was selected as the concentration for subsequent cuproptosis induction experiments. Furthermore, TTM rescue experiments demonstrated that the addition of TTM significantly

restored cell viability in both cell lines, indicating that the cytotoxic effect induced by ES-Cu was associated with copper accumulation (Fig. 3D and E). In addition, intracellular copper levels were measured in C666-1 cells following LIAS overexpression and ES-Cu treatment (Fig. 3F). The results showed that LIAS overexpression significantly increased intracellular copper accumulation, and this effect was further enhanced after ES-Cu treatment.

The fluorescence intensity of DLAT and phalloidin staining in LIAS overexpressing C666-1 cells was further examined (Fig. 3C). Compared with the ov-NC group, both the ov-LIAS group and ov-NC + ES-Cu group exhibited increased DLAT fluorescence intensity accompanied by decreased phalloidin positive areas. Furthermore, the ov-LIAS + ES-Cu group showed the highest DLAT fluorescence intensity and the lowest phalloidin positive area among all experimental groups (Fig. 3G and H). Western blot analysis was subsequently performed to evaluate cuproptosis-related proteins. Consistent with the immunofluorescence results, the ov-LIAS + ES-Cu group exhibited the highest DLAT protein expression and the lowest Lip-DLAT level among the four groups (Fig. 3I). Compared with the ov-NC group, FDX1 and LIAS expression levels showed a decreasing trend following ES-Cu treatment. In the ov-LIAS group, FDX1 expression was significantly decreased, whereas LIAS expression was increased. Following ES-Cu treatment, both FDX1 and LIAS protein levels were reduced in the ov-LIAS + ES-Cu group (Fig. 3J).

3.4 Identification of LHPP as a potential downstream mediator of LIAS in NPC cells

To further identify the downstream factors involved in LIAS-mediated regulation of cuproptosis in NPC cells, transcriptome sequencing was performed in C666-1 cells with LIAS overexpression. Compared with the ov-NC group, a total of 1,130 DEGs were identified in the ov-LIAS group, including 740 upregulated and 390 downregulated genes (Fig. 4A). Previous studies have demonstrated that tumor cells enhance glycolytic activity to generate sufficient energy to support rapid proliferation [13]. Therefore, an intersection analysis was performed between glycolysis-related gene sets and DEGs. Intersection analysis revealed 11 overlapping genes, including DDIT4, QSOX1, PPFIA4, CLDN3, ALDOA, ANG, LHPP, ISG20, CHST6, CAPN5, and HOMER1 (Fig. 4B). Among these candidates, LHPP was selected for further investigation due to its reported involvement in regulating tumor cell proliferation, migration, and metabolic homeostasis.

To investigate the potential interaction between LIAS and LHPP, molecular docking analysis was performed. The predicted docking model indicated that LIAS and LHPP could form a stable protein complex through multiple amino acid interactions (Fig. 4C). Several amino acid residues were predicted to participate in the LIAS-LHPP interaction, among these residues, TYR178 showed the strongest predicted binding contribution, with a binding energy of − 6.7 $kcal \cdot mol^{-1}$, followed by ARG157, which exhibited a binding energy of − 5.3 $kcal \cdot mol^{-1}$ (Fig. 4D). Furthermore, molecular dynamics simulation was conducted to evaluate the stability of the LIAS-LHPP complex. During the 100-ns simulation, the complex maintained a stable conformation, with the structures at different time points (0, 25, 50, 75, and

100 ns) showing minimal conformational changes (Fig. 4E). Additional analyses, including RMSD, RMSF, radius of gyration, hydrogen bonding, solvent accessible surface area (SASA), and free energy landscape analysis, further supported the stability of the LIAS-LHPP interaction (Figure S2).

3.5 LIAS regulates LHPP expression and interacts with LHPP in NPC cells

To further examine the relationship between LIAS and LHPP, LHPP protein expression in C666-1 cells was detected by Western blot following LIAS overexpression and ES-Cu treatment. Compared with the ov-NC group, LHPP protein levels showed no significant change in the ov-NC + ES-Cu group, whereas LHPP expression was significantly increased in the ov-LIAS group. Notably, LHPP protein levels were further increased in the ov-LIAS + ES-Cu group compared with the ov-LIAS group (Fig. 5A). To further investigate the interaction between LIAS and LHPP, Co-IP assays were performed. In the IP group, LHPP was efficiently enriched following immunoprecipitation with an anti-LHPP antibody, whereas no obvious enrichment was detected in the IgG control group. LIAS was also detected in the IP samples (Fig. 5B). Reciprocal Co-IP using an anti-LIAS antibody showed enrichment of LIAS together with LHPP in the immunoprecipitated complex, while neither protein showed obvious enrichment in the IgG control group (Fig. 5C). The cellular distribution of LIAS and LHPP was further examined by immunofluorescence staining. In untreated C666-1 cells, LIAS positive cells also exhibited LHPP fluorescence, with substantial overlap between the LIAS and LHPP signals (Fig. 5D).

3.6 LIAS interacts with LHPP and inhibits glycolytic metabolism in NPC cells

To further investigate the relationship between LIAS and LHPP, plasmids expressing wild-type LHPP (ov-LHPP^{WT}) and mutant LHPP (ov-LHPP^{MUT}), in which arginine at position 157 was substituted with alanine, were constructed based on the predicted interaction between LIAS and LHPP and subsequently transfected into C666-1 cells. Western blot analysis showed that LHPP protein expression was significantly increased in the ov-LIAS + ov-NC group compared with the ov-NC + ov-NC group. Compared with ov-LIAS + ov-NC, LHPP expression was further increased following ov-LHPP^{WT} overexpression, whereas only a nonsignificant increasing trend was observed in the ov-LIAS + ov-LHPP^{MUT} group. In contrast, LIAS protein levels were significantly higher in all three LIAS overexpressing groups than in the ov-NC + ov-NC group, with no significant differences among the ov-LIAS + ov-NC, ov-LIAS + ov-LHPP^{WT}, and ov-LIAS + ov-LHPP^{MUT} groups (Fig. 6A). To further examine the interaction between LIAS and LHPP, Co-IP assays were performed in C666-1 cells overexpressing LIAS and LHPP. In the IP group, a clear Flag-LIAS band was detected, whereas no obvious signal was observed in the IgG control group. HA-LHPP was also detected in the Flag-LIAS immunoprecipitated complex, while no obvious HA-LHPP enrichment was detected in the IgG control group (Fig. 6B).

The cellular localization of LIAS and LHPP was further evaluated by immunofluorescence staining. LIAS overexpression markedly increased LHPP fluorescence intensity compared with the ov-NC + ov-NC group, whereas LHPP overexpression did not significantly alter LIAS fluorescence intensity. Notably, the LIAS and LHPP fluorescence signals showed the greatest overlap in the ov-LIAS + ov-LHPP^{WT} group, while this colocalization was significantly reduced in the ov-LIAS + ov-LHPP^{MUT} group (Fig. 6C). To determine whether the LIAS-LHPP interaction was associated with changes in cellular energy metabolism, pyruvate, ATP, glucose consumption, and lactate production were subsequently measured. Compared with the ov-NC + ov-NC group, LIAS overexpression significantly reduced all four metabolic parameters. These parameters were further reduced in the ov-LIAS + ov-LHPP^{WT} group, whereas partial recovery was observed following ov-LHPP^{MUT} overexpression compared with the ov-LIAS + ov-LHPP^{WT} group (Fig. 6D-G).

3.7 LHPP overexpression enhances the inhibitory effects of LIAS on NPC cell malignant phenotypes and cuproptosis

To further investigate the functional role of the LIAS-LHPP axis in ES-Cu induced cuproptosis, the effects of LHPP overexpression or mutation on the malignant phenotypes of C666-1 cells were first evaluated. CCK-8 assays showed that cell viability was lowest in the ov-LIAS + ES-Cu + ov-LHPP^{WT} group, whereas viability was partially restored in the ov-LIAS + ES-Cu + ov-LHPP^{MUT} group (Fig. 7A). Consistently, wound healing assays showed that the ov-LIAS + ES-Cu + ov-LHPP^{WT} group exhibited the lowest migratory capacity, while migration was partially restored following LHPP mutation (Fig. 7B and C). Transwell assays further showed that both the number of migrated and invaded cells was lowest in the ov-LIAS + ES-Cu + ov-LHPP^{WT} group, whereas both phenotypes were significantly increased in the ov-LIAS + ES-Cu + ov-LHPP^{MUT} group (Fig. 7C-E).

The effects of LHPP on cuproptosis associated cellular changes were subsequently examined by immunofluorescence staining. DLAT fluorescence intensity was highest in the ov-LIAS + ES-Cu + ov-LHPP^{WT} group, whereas the signal was reduced in the ov-LIAS + ES-Cu + ov-LHPP^{MUT} group. Phalloidin staining further showed more pronounced disruption of the actin cytoskeleton in the ov-LIAS + ES-Cu + ov-LHPP^{WT} group, while partial preservation of cytoskeletal structures was observed following LHPP mutation (Fig. 7F-H). To further characterize the effects of LHPP on cuproptosis-related proteins, the expression levels of were examined by Western blot. LIAS, LHPP, and DLAT protein levels were higher in both the ov-LIAS + ES-Cu + ov-LHPP^{WT} and ov-LIAS + ES-Cu + ov-LHPP^{MUT} groups than in the ov-LIAS + ES-Cu group, with no significant differences between these two groups. In contrast, FDX1 and Lip-DLAT protein levels were lower in both LHPP overexpressing groups than in the ov-LIAS + ES-Cu group, with no significant difference between the two LHPP overexpressing groups (Fig. 7I).

3.8 LIAS overexpression enhances the anti-tumor effects of Elesclomol *in vivo*

Following the *in vitro* findings that the LIAS-LHPP axis enhanced ES-Cu induced cytotoxicity and suppressed the malignant phenotypes of NPC cells, the effects of LIAS overexpression and Elesclomol treatment were examined *in vivo*. Tumor volume and tumor weight were monitored to assess tumor growth. Compared with the ov-NC group, LIAS overexpression significantly reduced tumor volume, while the combination of LIAS overexpression and Elesclomol treatment resulted in a further reduction in tumor volume (Fig. 8C). Consistently, tumor weight showed a similar pattern, with the ov-LIAS+Elesclomol group exhibiting the lowest tumor weight (Fig. 8D).

H&E staining showed increased tumor cell necrosis in all treatment groups compared with the ov-NC group, with the most extensive necrotic areas observed in the ov-LIAS+Elesclomol group (Fig. 8A and E). Consistent with these histological changes, IHC analysis showed that the proportion of Ki-67 positive areas was significantly decreased in all treatment groups, with the lowest Ki-67 expression observed in the ov-LIAS+Elesclomol group (Fig. 8B and F). To characterize the metabolic changes associated with LIAS overexpression and Elesclomol treatment *in vivo*, glycolysis related metabolic parameters were subsequently analyzed. Compared with the ov-NC group, pyruvate levels, ATP levels, glucose consumption, and lactate production were significantly decreased in both the ov-NC+Elesclomol and ov-LIAS groups (Fig. 8G-J). Notably, all four parameters were further reduced in the ov-LIAS+Elesclomol group.

3.9 LIAS overexpression enhances ES-Cu induced cuproptosis *in vivo*

Following the above findings that LIAS overexpression enhanced the anti-tumor effects of Elesclomol *in vivo*, the copper accumulation and cuproptosis-related molecular changes in tumor tissues were then examined. Compared with the ov-NC group, copper content was significantly increased in both the ov-NC+Elesclomol and ov-LIAS groups. Moreover, copper content was further increased in the ov-LIAS+Elesclomol group compared with either the ov-NC+Elesclomol or ov-LIAS group (Fig. 9A). The expression and co-localization of LIAS and LHPP in tumor tissues were subsequently examined by immunofluorescence staining (Fig. 9C). Compared with the ov-NC group, LIAS fluorescence intensity was significantly increased in the ov-LIAS group, whereas it was decreased in the ov-NC+Elesclomol group. Compared with the ov-LIAS group, LIAS fluorescence intensity was further decreased in the ov-LIAS+Elesclomol group. In contrast, LHPP fluorescence intensity was significantly increased in the ov-LIAS group but showed no significant change in the ov-NC+Elesclomol group compared with the ov-NC group. Notably, LHPP fluorescence intensity was highest in the ov-LIAS+Elesclomol group (Fig. 9B). Analysis of the LIAS and LHPP co-localization signal showed a significant increase in the ov-LIAS group compared with the ov-NC group, whereas no significant difference was observed between the ov-NC+Elesclomol and ov-NC groups. The strongest LIAS and LHPP co-localization signal was observed in the ov-LIAS+Elesclomol group (Fig. 9B).

Cuproptosis-related protein expression was subsequently analyzed in tumor tissues. Compared with the ov-NC group, DLAT protein expression was significantly increased in both the ov-NC+Elesclomol and ov-

LIAS groups and was further increased in the ov-LIAS+Elesclomol group. In contrast, FDX1 and Lip-DLAT protein levels were significantly decreased in both the ov-NC+Elesclomol and ov-LIAS groups, with the lowest levels observed in the ov-LIAS+Elesclomol group (Fig. 9E). Western blot analysis further showed that LIAS and LHPP protein levels were significantly increased in the ov-LIAS group compared with the ov-NC group, whereas neither protein showed a significant increase in the ov-NC+Elesclomol group. In the ov-LIAS+Elesclomol group, LHPP expression was further increased, whereas LIAS expression was significantly lower than that in the ov-LIAS group (Fig. 9D).

4 Disscussion

Heavy metal ions function as essential micronutrients, but dysregulated levels can induce cell death [14]. Among these ions, copper (Cu) is crucial for a wide range of biological processes, serving as a co-factor for numerous metabolic enzymes. Cuproptosis is a copper-dependent form of regulated cell death that has garnered growing interest for its role in tumor initiation and progression. Studies have shown that elevated intracellular Cu concentrations is related to cancer progression [15–17]. And as the degree of cell differentiation changes in NPC, metabolic processes such as lipid metabolism, amino acid metabolism, and tricarboxylic acid (TCA) cycle will also undergo significant changes [18]. Therefore, it is speculated that there may be a certain regulatory relationship between Cu and NPC.

LIAS belongs to the biotin and lipoic acid synthase family and is an important CRG that primarily catalyzes the final step in lipoic acid biosynthesis [19]. Among the CRGs, LIAS is critical for modulating mitochondrial energy metabolism and maintaining cellular antioxidant defenses [20]. Studies have shown that the LIAS gene exhibits differential expression across various cancers, and its expression may serve as a potential indicator for predicting the efficacy of immunotherapy in cancer patients [21]. NPC is the most common subtype of head and neck squamous cell carcinoma. Previous studies have shown that among patients with head and neck squamous cell carcinoma (HNSCC), those with high Jab1 expression exhibit elevated LIAS expression levels and have better prognosis [22]. However, current research on the role of LIAS in NPC remains relatively limited. Therefore, this study integrated bioinformatics analyses with *in vitro* and *in vivo* experiments to characterize LIAS expression in NPC and its effects on malignant tumor behaviors. We further investigated the downstream mechanisms of LIAS and their involvement in cellular energy metabolism and cuproptosis.

Studies have shown that the expression levels of certain genes are closely associated with the prognosis of patients with NPC. For example, high expression of STOML1 is associated with longer disease-free survival in NPC patients [23], while positive expression of IGFBP6 significantly reduces the risk of local recurrence and distant metastasis [24]. Consistent with these findings, IHC and RT-qPCR analyses of clinical samples demonstrated that LIAS expression was significantly lower in NPC tissues than in BNPL tissues, and a trend toward higher LIAS expression was observed in early stage NPC patients. In addition, cell-based analyses showed that LIAS expression was consistently reduced in both NPC cell lines examined. Overall, these findings indicate that LIAS is closely associated with the development and progression of NPC.

NPC is characterized by strong local invasiveness and a high incidence of cervical lymph node metastasis, and some patients may still develop distant metastasis after treatment [25]. Enhanced migration and invasion of tumor cells are important biological bases underlying local invasion and distant metastasis. Therefore, investigating the effects of LIAS on the proliferation, migration, and invasion of NPC cells is of considerable significance. Previous studies have demonstrated that LIAS expression was downregulated in both prostate cancer and cuproptosis models [10]. Overexpression of LIAS in LNCaP cells significantly reduced tumor cell viability, and LIAS exerted an antitumor effect against prostate cancer by enhancing cuproptosis. In this study, LIAS knockdown significantly increased cell viability, whereas LIAS overexpression significantly suppressed cell viability, suggesting that LIAS may negatively regulate the proliferation of NPC cells. SLC31A1 is a key gene involved in cuproptosis. Existing studies have found that SLC31A1 was overexpressed in breast cancer and was associated with poor prognosis. Knockdown of SLC31A1 reduced the proliferation, migration, and invasion of breast cancer cells and effectively decreased tumor volume in a mouse model [26]. Consistent with these findings, LIAS knockdown significantly enhanced the migration and invasion of NPC cells, whereas LIAS overexpression significantly suppressed these behaviors in the present study. Moreover, LIAS overexpression significantly reduced both tumor volume and tumor weight in the xenograft model. Collectively, these findings indicate that LIAS exerts a tumor suppressive role in NPC.

Mitochondria are not only a major site of cellular energy metabolism but also a key organelle involved in cuproptosis. Cuproptosis is highly dependent on mitochondrial oxidative metabolism. Upon copper stimulation, the homeostasis of lipoylated proteins in mitochondria is disrupted, which may further affect the TCA cycle and cellular energy metabolism [13]. Further experiments showed that LIAS overexpression significantly reduced intracellular pyruvate and ATP levels, while decreasing glucose consumption and lactate production, suggesting that LIAS may suppress the metabolic activity of NPC cells. These findings were further supported by the *in vivo* experiments, in which LIAS overexpression and Elesclomol treatment both reduced pyruvate, ATP, glucose consumption, and lactate levels in xenograft tumors, with the greatest reductions observed in the combination treatment group. Collectively, these findings suggest that LIAS may affect the response of NPC cells to Elesclomol by regulating cellular energy metabolism.

Further investigations were conducted to determine whether LIAS enhances cuproptosis in NPC cells. In C666-1 cells, LIAS overexpression further increased cellular copper levels in the presence of ES-Cu. TTM is a copper ion chelator [27]. In this study, TTM significantly reversed the reduction in cell viability induced by LIAS overexpression and ES-Cu treatment. These findings indicated that LIAS increased the sensitivity of NPC cells to cuproptosis inducers and consequently exerted an anti-tumor effect. Previous studies have demonstrated that cancer cells attempt to resist cuproptosis by inhibiting the lipoylation process of proteins catalyzed by FDX1 and LIAS, thereby reducing the accumulation of lipoylated proteins [13, 28, 29]. Thus, the depletion of these proteins has also been widely considered as a molecular indicator for evaluating the occurrence and extent of cuproptosis. In this study, LIAS overexpression combined with ES-Cu treatment significantly decreased the protein levels of FDX1, LIAS, and Lip-DLAT, while increasing the expression of total DLAT, suggesting that LIAS promotes cuproptosis

in NPC cells. F-actin is an essential component of the cytoskeleton and serves as a fundamental structure supporting the migratory and invasive abilities of tumor cells. Phalloidin can specifically bind to F-actin, and is widely used as a fluorescent probe to investigate cytoskeletal remodeling and malignant biological behaviors in cancers such as breast cancer, thyroid carcinoma, and colorectal cancer [30–32]. Previous studies have shown that silencing Piezo1 reduces the phalloidin positive area in cervical cancer cells, suggesting disruption of the cytoskeletal structure [33]. In this study, LIAS overexpression combined with ES-Cu treatment aggravated the degree of cytoskeletal disruption. Together, these findings support the involvement of cuproptosis in the inhibitory effects of LIAS on NPC cells.

Transcriptomic analysis was further performed to identify potential downstream regulators of LIAS, and intersection analysis identified LHPP and other candidate genes. LHPP is a histidine phosphatase that is closely associated with cell survival, growth, and differentiation. Previous studies have shown that LHPP exerts a tumor suppressive role in hepatocellular carcinoma, and that low LHPP expression is associated with increased tumor severity and shorter overall survival in patients [34]. In addition, LHPP has been shown to exert tumor suppressive effects in colorectal cancer, pancreatic cancer, gastric cancer, and oral squamous cell carcinoma by regulating cell proliferation and metastasis, impairing energy metabolism, and promoting apoptosis [35–38]. Therefore, LHPP was further investigated as a potential downstream effector of LIAS in the present study. The results showed that LIAS overexpression increased LHPP expression, and that LIAS interacted with LHPP, suggesting that LHPP may participate in the biological effects mediated by LIAS. Functional experiments further demonstrated that additional LHPP overexpression in the context of LIAS overexpression further suppressed the proliferation, migration, and invasion of NPC cells, accompanied by further reductions in pyruvate, ATP, glucose consumption, and lactate levels, suggesting that LHPP may contribute to the regulation of malignant biological behaviors and energy metabolism by LIAS in NPC. In addition, LHPP overexpression further enhanced the cytotoxicity induced by ES-Cu treatment and promoted DLAT related changes, accompanied by further reductions in FDX1 and Lip-DLAT levels. These effects were partially reversed following LHPP mutation, suggesting that LHPP may participate in the regulation of LIAS mediated sensitivity to cuproptosis. Molecular docking and molecular dynamics simulations further indicated that LIAS and LHPP may form a relatively stable interaction complex, and that the R157 residue of LHPP may contribute to their interaction. However, the precise molecular mechanism requires further experimental validation. Collectively, these findings suggest that LHPP may represent an important downstream effector of LIAS in regulating malignant biological behaviors, energy metabolism, and cuproptosis in NPC, and that the LIAS–LHPP axis may constitute a key regulatory link connecting metabolic reprogramming with cuproptosis sensitivity in NPC.

This study has several limitations. First, although the R157A mutation of LHPP attenuated some of the biological effects and cuproptosis-related phenotypes, the specific role of the R157 residue in the interaction between LIAS and LHPP requires further experimental validation. Second, this study mainly evaluated the metabolic effects of the LIAS-LHPP axis using energy metabolism related indicators. Future studies should incorporate mitochondrial function, oxidative phosphorylation, and the TCA cycle to further elucidate its effects on mitochondrial metabolism. Finally, although copper levels, FDX1, DLAT,

and Lip-DLAT were assessed to evaluate cuproptosis, additional cuproptosis-related metabolic and molecular indicators are needed to further clarify the mechanisms by which the LIAS-LHPP axis regulates cuproptosis.

Methodologically, this study used an integrated workflow. This workflow included transcriptomic screening, molecular docking, dynamics simulation, co-immunoprecipitation validation, and mutant-based functional verification. It provides a practical framework for identifying and validating cuproptosis-related protein–protein interactions. First, transcriptomic profiling was used to prioritize candidate downstream effectors. Second, computational prediction identified interaction interfaces and key residues. Third, experimental confirmation was performed by co-immunoprecipitation and functional rescue with site-directed mutants. This sequential strategy reduces false positives and strengthens causal inference. This approach may be applicable to other cuproptosis-related proteins and cancer models. It may also facilitate the discovery of novel molecular axes that link metabolic reprogramming to cell death sensitivity.

5 Conclusion

In conclusion, LIAS was downregulated in NPC and exerted tumor suppressive effects by inhibiting malignant biological behaviors and altering cellular energy metabolism. Mechanistically, LIAS interacted with and positively regulated LHPP, which contributed to the inhibitory effects of LIAS on NPC cell proliferation, migration, and invasion. Moreover, the LIAS-LHPP axis altered the metabolic state of NPC cells and enhanced their sensitivity to ES-Cu induced cuproptosis. *In vivo* experiments further supported the antitumor effects of LIAS overexpression and its synergistic effects with ES-Cu. Collectively, these findings suggest that LIAS may be involved in regulating metabolic reprogramming and cuproptosis sensitivity in NPC, and provide a potential molecular basis for targeting cuproptosis in NPC.

Declarations

Ethics approval and consent to participate

This study was conducted in accordance with the Declaration of Helsinki. The protocol for the retrospective analysis of archived, anonymized specimens was approved by the Ethics Committee of Chongqing General Hospital (Approval No. IIT S2025-044-01), which waived the requirement for individual written informed consent due to the use of pre-existing, de-identified materials. For any prospectively collected samples or data, written informed consent was obtained from all participants. All animal experiments were approved by the Ethics Committee of Chongqing General Hospital (Approval No. KY S2025-019-01) and performed in accordance with the ARRIVE guidelines and relevant national guidelines for animal welfare. Clinical trial registration is not applicable as this is not an interventional study.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Author Contribution

Hongjiang Chen: Conceptualization, Methodology, Investigation, Formal Analysis, Writing – Original Draft, Data Curation. Xu Jiang: Conceptualization, Methodology, Writing – Original Draft, Investigation, Data Curation. Yaqin Hu: Investigation, Data Curation, Validation. Ting Yang: Investigation, Data Curation. Wei Yuan: Supervision, Funding Acquisition, Writing – Review & Editing, Project Administration. All authors read and approved the final manuscript.

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Data Availability

All data generated or analysed during this study are included in this published article and its supplementary information files, except for the raw transcriptomic sequencing data, which are available from the corresponding author on reasonable request.

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